Substrate Specificity of the *Escherichia coli* Endonuclease III: Excision of Thymine- and Cytosine-Derived Lesions in DNA Produced by Radiation-Generated Free Radicals†

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ABSTRACT: The excision of modified bases from DNA by *Escherichia coli* endonuclease III was investigated. Modified bases were produced in DNA by exposure of dilute buffered solutions of DNA to ionizing radiation under oxic or anoxic conditions. The technique of gas chromatography/mass spectrometry (GC/MS) was used to identify and quantify 16 pyrimidine- and purine-derived DNA lesions. DNA substrates were incubated either with the native enzyme or with the heat-inactivated enzyme. Subsequently, DNA was precipitated. Pellets were analyzed by GC/MS after hydrolysis and derivatization. Supernatant fractions were analyzed after derivatization without hydrolysis. The results provided unequivocal evidence for the excision by *E. coli* endonuclease III of a number of thymine- and cytosine-derived lesions from DNA. These were 5,6-dihydrothymine, 5-hydroxy-5-methylhydantoin, thymine glycol, 5-hydroxy-6-hydrothymine, 5,6-dihydrouracil, alloxan, uracil glycol, and 5-hydroxy-6-hydrouracil. None of the purine-derived lesions was excised by endonuclease III. The present work extends the substrate specificity of *E. coli* endonuclease III to another thymine-derived and four cytosine-derived lesions. It is the first investigation of the substrate specificity of this repair enzyme in the context of a large number of pyrimidine- and purine-derived lesions in DNA.

Generation of oxygen-derived species by endogenous and exogenous sources may lead to DNA damage in living systems by a variety of mechanisms [for a review see Halliwell and Gutteridge (1989)]. Oxygen-derived species have been suggested to play an important role in biological processes such as mutagenesis, carcinogenesis, reproductive cell death, and aging [for reviews see Halliwell and Gutteridge (1989), Breimer (1990), Lindahl (1993)]. Superoxide radical (O_2^-)[1] and H_2O_2 are generated in all aerobic cells (Fridovich, 1986; Halliwell & Gutteridge, 1989). However, neither O_2^- nor H_2O_2 by itself causes any DNA damage (Lesko et al., 1980; Sagripanti & Kraemer, 1989; Aruoma et al., 1989; Blakely et al., 1990). In metal ion-catalyzed reactions, these species may be converted into hydroxyl radical (•OH), which reacts with organic compounds including DNA components at or near diffusion-controlled rates [for reviews see von Sonntag (1987) and Halliwell and Gutteridge (1989)]. Hydroxyl radical and other radical species [H atom, hydrated electron (e_{aq}^-)] are also formed in living cells by interaction of ionizing radiation with cellular water [for a review see von Sonntag (1987)]. Hydroxyl radical produces a unique pattern of modifications in DNA and chromatin such as modified bases and sugars, single-strand breaks, double-strand breaks, abasic sites, and DNA–protein cross-links [for reviews see von Sonntag (1987), Oleinick et al. (1987), Steenken (1989), and Dizdaroglu (1991)].

Modifications in DNA are subject to cellular repair processes in vivo and may be removed from DNA by specific repair enzymes [for reviews see Wallace (1988), Sancar and Sancar (1988), and Ramotar and Demple (1993)]. Failure of cells to repair DNA damage may result in deleterious biological consequences [for a review see Breimer (1990)]. Of the repair enzymes isolated and characterized thus far, endonuclease III from *Escherichia coli* isolated by Radman (1976) has been shown to possess both a DNA *N*-glycosylase activity and an abasic (apurinic and/or apyrimidinic) site nicking activity (Demple & Linn, 1980; Breimer & Lindahl, 1984; Cunningham & Weiss, 1985; Asahara et al., 1989). The *nth* gene coding for endonuclease III has been cloned and sequenced (Cunningham & Weiss, 1985; Asahara et al., 1989). Enzymes with substrate specificity and mechanism of action similar to those of *E. coli* endonuclease III have been identified in bacteria (Jorgensen et al., 1987), yeast (Gosset et al., 1988), and mammalian cells and tissues (Helland et al., 1986; Doetsch et al., 1986, 1987; Lee et al., 1987; Huq et al., 1992). Endonuclease III has been shown to incise damaged DNA at

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[1] Abbreviations: O_2^-, superoxide radical; •OH, hydroxyl radical; 5,6-diHThy, 5,6-dihydrothymine; 5,6-diHUra, 5,6-dihydrouracil; 5,6-diHCyt, 5,6-dihydrocytosine; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OH-Ura, 5-hydroxyuracil; 5-OH-Cyt, 5-hydroxycytosine; 5-OH-6-HThy, 5-hydroxy-6-hydrothymine; 5-OH-6-HCyt, 5-hydroxy-6-hydrocytosine; 5-OH-6-HUra, 5-hydroxy-6-hydrouracil; 5-OHMeUra, 5-(hydroxymethyl)uracil; Cyt, cytosine; Thy, thymine; Ura, uracil; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; 5,6-diOH-Ura, 5,6-dihydroxyuracil; FapyAde, 4,6-diamino-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 2-OH-Ade, 2-hydroxyadenine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Gua, 8-hydroxyguanine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; GC/MS-SIM, gas chromatography/mass spectrometry with selected-ion monitoring; Gy, gray ($J \cdot kg^{-1}$); nd, not detected.

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cytosine and guanine sites, without identification of lesions involved (Doetsch et al., 1986, 1987; Helland et al., 1986; Weiss & Duker, 1986, 1987). The *N*-glycosylase activity of endonuclease III removes a number of pyrimidine lesions from double-stranded DNA damaged by free radicals, oxidizing agents, and ultraviolet radiation (Demple & Linn, 1980; Breimer & Lindahl, 1980, 1984, 1985; Katcher & Wallace, 1983; Doetsch et al., 1986; Weiss & Duker, 1986; Helland et al., 1986; Higgins et al., 1987; Laspia & Wallace, 1988; Boorstein et al., 1989; Ganguly et al., 1990). These lesions include urea, 5,6-dihydrothymine, thymine glycol, 5-hydroxy-5-methylhydantoin, methyltartronylurea, an unidentified hydroxyhydrothymine, an unidentified cytosine photoproduct, and thymine, cytosine, and uracil hydrates. Because of the analytical techniques used, the excision of only one or a limited number of lesions has been assessed at a time. Thus, a broad substrate specificity of this enzyme covering both pyrimidine- and purine-derived modified DNA bases has not been investigated. Recently, we have studied the substrate specificity of the Fpg protein from *E. coli* (formamidopyrimidine-DNA glycosylase) using DNA substrates damaged by free radicals or by photosensitization (Boiteux et al., 1992). The technique of gas chromatography/mass spectrometry (GC/MS) was used to assess the ability of the Fpg protein to excise both pyrimidine and purine lesions from DNA. This technique is well suited for identification and quantification of a large number of base lesions in the same sample of DNA [for a review see Dizdaroglu (1991)]. Thus, it permits precise determination of excision (or nonexcision) by DNA glycosylases of both pyrimidine and purine lesions from DNA.

In the present work, we have studied the excision by *E. coli* endonuclease III of pyrimidine- and purine-derived lesions from DNA, which was damaged by ionizing radiation-generated free radicals in the absence or presence of oxygen.

EXPERIMENTAL PROCEDURES

Materials.[2] Calf thymus DNA, 5,6-dihydrothymine (5,6-diHThy), 5,6-dihydrouracil (5,6-diHUra), isobarbituric acid [5-hydroxyuracil (5-OH-Ura)], 5-(hydroxymethyl)uracil (5-OHMeUra), alloxan [2,4,5,6(1*H*,3*H*)-pyrimidinetetrone], isoguanine [2-hydroxyadenine (2-OH-Ade)], and 6-azathymine were purchased from Sigma Chemical Co. 5-Hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 5-hydroxycytosine (5-OH-Cyt), 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine (8-OH-Ade), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), 8-hydroxyguanine (8-OH-Gua), dialuric acid, 4,6-diamino-5-formamidopyrimidine-*1,3-*$^{15}N_2$-*2-*^{13}C-(*5-aminoformyl-*^{15}N,^{2}H), 8-hydroxyadenine-*1,3,7-*$^{15}N_3$-*2,8-*$^{13}C_2$, 2,6-diamino-4-hydroxy-5-formamidopyrimidine-*1,3-*$^{15}N_2$-(*5-amino-*^{15}N)-*2-*^{13}C, 8-hydroxyguanine-*1,3-*$^{15}N_2$-(*2-amino-*^{15}N)-*2-*^{13}C, dialuric acid-*1,3-*$^{15}N_2$-*2,4-*$^{13}C_2$, 5-hydroxy-5-methylhydantoin-*1,3-*$^{15}N_2$-*2-*^{13}C, and 5-hydroxycytosine-*1,3-*$^{15}N_2$-*2-*^{13}C were purchased from Program Resources, Inc./Dyncorp, National Cancer Institute-FCRD (Frederick, MD). Thymine-$\alpha,\alpha,\alpha,6$-$^{2}H_4$ and *cis*-thymine glycol-$\alpha,\alpha,\alpha,6$-$^{2}H_4$ were obtained from Merck & Co. Inc./Isotopes. *cis*-Thymine glycol was a gift from Dr. W. F. Blakely of the Armed Forces Radiobiology Research Institute, Bethesda, MD. Alloxan-*1,3-*$^{15}N_2$-*2,4-*$^{13}C_2$ was obtained by dissolving dialuric acid-*1,3-*$^{15}N_2$-*2,4-*$^{13}C_2$ in water (Behrend & Roosen, 1889; Dizdaroglu, 1993). 5-Hydroxyhydantoin (5-OH-Hyd) and 5-hydroxyhydantoin-*1,3-*$^{15}N_2$-*2,4-*$^{13}C_2$ were synthesized by treatment of dialuric acid and dialuric acid-*1,3-*$^{15}N_2$-*2,4-*$^{13}C_2$, respectively, with 88% formic acid at 150 °C for 1 h (Dizdaroglu, 1993). 5-Hydroxy-6-hydrothymine (5-OH-6-HThy) and 5-hydroxy-6-hydrouracil (5-OH-6-HUra) were not available. They were identified on the basis of the known mass spectra and gas chromatographic behaviors of their trimethylsilyl derivatives (Dizdaroglu, 1984, 1985). Formic acid was purchased from Mallinckrodt. Acetonitrile and bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% (trimethylsilyl)chlorosilane were obtained from Pierce Chemical Co. Dialysis membranes with a molecular weight cutoff of 6000–8000 were obtained from Fisher Scientific Co.

Irradiations. Calf thymus DNA was dissolved in 10 mM phosphate buffer (pH 7.4) (0.25 mg of DNA/mL) and then dialyzed extensively against 10 mM phosphate buffer. Subsequently, DNA solutions were bubbled with nitrogen or air for 30 min and then irradiated in a ^{60}Co γ-source (dose rate 70 Gy/min). The total radiation dose applied was 55 Gy. After irradiation, samples were dialyzed against 10 mM phosphate buffer (pH 7.4). Aliquots of unirradiated and irradiated DNA samples were dried in a SpeedVac under vacuum at room temperature.

Purification of Endonuclease III. The endonuclease III protein was isolated from an overproducing *E. coli* strain BH410 (as JM105 but fpg-1:Kn) (Tudek et al., 1993) harboring the pNTH10 plasmid. This plasmid contained the *nth* gene placed under the control of the *lac* promoter and was a subclone of the pRPC53 plasmid (Cunningham & Weiss, 1985) in pUC18 plasmid vector. The endonuclease III was purified to apparent homogeneity from 14 g (wet weight) of BH410/pNTH10 bacteria induced for 4 h in the presence of 0.5 mM IPTG. All purification steps were similar to those described for the purification of the Fpg protein (Boiteux et al., 1990), but fraction IV was supplemented with 1.5 M ammonium sulfate and the active fractions were eluted at 0.8 M ammonium sulfate (fraction V). The protein concentration of fraction V was 0.72 mg/mL determined by the method of Bradford. The enzyme activity was measured using an [^{3}H]-thymine DNA of *E. coli* (62 270 cpm/μg) containing apurinic sites as substrate (Boiteux et al., 1990).

Enzymatic Assays. The standard reaction mixture (100 μL final volume) contained 50 mM phosphate buffer (pH 7.4), 100 mM KCl, 60 μg of DNA, and 5 μg of endonuclease III (fraction V). When indicated (boiled enzyme), endonuclease III was inactivated by heat treatment at 90 °C for 10 min. Reaction mixtures were incubated at 37 °C for 20 min. Subsequently, 250 μL of cold ethanol (–20 °C) was added to each sample, and the resulting mixture was kept at –20 °C for 30 min. The mixture was centrifuged at 4 °C for 15 min in an Eppendorf microfuge. The precipitated DNA (pellet) and the supernatant fraction were separated and dried in a SpeedVac under vacuum at room temperature.

Hydrolysis and Derivatization. Pellets were dissolved in water, and the absorbance at 260 nm of each sample was measured to calculate the amount of DNA (absorbance of 1 = 50 μg of DNA/mL). To supernatant fractions and to $\approx$30 μg of DNA pellets were added 2 nmol of thymine-$\alpha,\alpha,\alpha,6$-$^{2}H_4$ and 0.5 nmol each of 6-azathymine, dialuric acid-*1,3-*$^{15}N_2$-*2,4-*$^{13}C_2$, *cis*-thymine glycol-$\alpha,\alpha,\alpha,6$-$^{2}H_4$, 5-hydroxy-5-methylhydantoin-*1,3-*$^{15}N_2$-*2-*^{13}C, 5-hydroxycytosine-*1,3-*$^{15}N_2$-*2-*^{13}C, 4,6-diamino-5-formamidopyrimidine-*1,3-*$^{15}N_2$-*2-*^{13}C-(*5-aminoformyl-*^{15}N,^{2}H), 8-hydroxyadenine-*1,3,7-*$^{15}N_3$-*2,8-*$^{13}C_2$, 2,6-diamino-4-hydroxy-5-formamidopyrimidine-*1,3-*$^{15}N_2$-(*5-*

[2] Certain commercial equipment and materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials and equipment identified are necessarily the best available for the purpose.

amino-^{15}N)-2-^{13}C, 8-hydroxyguanine-1,3-$^{15}N_2$-(2-*amino*-^{15}N)-2-^{13}C as internal standards. Samples were then lyophilized. Dried pellets were hydrolyzed with 0.5 mL of formic acid (60%) in evacuated and sealed tubes for 30 min at 140 °C (Nackerdien et al., 1992). This was followed by lyophilization. Dried supernatant fractions were not hydrolyzed. Subsequently, dried supernatant fractions and hydrolyzed pellets were trimethylsilylated in polytetrafluoroethylene-capped hypovials (Pierce Chemical Co.) with 100 μL of a mixture of BSTFA and acetonitrile (4/1 v/v) by heating for 30 min at 130 °C.

Gas Chromatography/Mass Spectrometry (GC/MS). Analysis of derivatized samples by GC/MS with selected-ion monitoring (SIM) was performed as described previously (Boiteux et al., 1992). Quantification of 5-OH-Hyd, alloxan, Thy glycol, 5-OH-5-MeHyd, 5-OH-Cyt, FapyAde, 8-OH-Ade, FapyGua, and 8-OH-Gua was done by isotope dilution mass spectrometry using their stable isotope-labeled analogues as internal standards (Dizdaroglu, 1993). Other pyrimidine lesions, the labeled analogues of which were not available, were quantified using 6-azathymine as an internal standard (Fuciarelli et al., 1989; Nackerdien et al., 1992). 8-Hydroxyadenine-1,3,7-$^{15}N_3$-2,8-$^{13}C_2$ was also used as an internal standard to quantify 2-OH-Ade because of the similarity of the structures and mass spectra of these compounds. Thymine-$\alpha,\alpha,\alpha,6$-$^2H_4$ was used to assess the amount of thymine (thus DNA) in hydrolyzed DNA samples (Djuric et al., 1991; Dizdaroglu, 1993).

RESULTS

The objective of this work was to study the excision of various modified bases from DNA by *E. coli* endonuclease III. Modified bases in DNA were generated by exposure of dilute DNA solutions to ionizing radiation in the presence or absence of oxygen. Hydroxyl radical, H atom, and e_{aq}^- are produced when ionizing radiation interacts with water of dilute solutions [for a review see von Sonntag (1987)]. In the presence of oxygen, H atom and e_{aq}^- react with oxygen at diffusion-controlled rates and are converted into O_2^-. In the absence of oxygen, the yields of •OH, H atom, and e_{aq}^- amount to 0.28, 0.057, and 0.27 μmol/J, respectively. In air-saturated solutions, the yields of •OH and O_2^- are 0.28 and 0.33 μmol/J, respectively. The types of modified bases and their yields in irradiated DNA or in mammalian chromatin substantially depend on the presence of oxygen (Fuciarelli et al., 1990; Gajewski et al., 1990).

Aliquots of irradiated DNA samples were incubated with either the native enzyme or the heat-inactivated enzyme and subsequently ethanol-precipitated. As controls, other aliquots of DNA samples were precipitated in the incubation buffer without prior treatment with the enzyme. All pellets were analyzed by GC/MS-SIM after acidic hydrolysis and trimethylsilylation. The supernatant fractions of the same samples were analyzed after lyophilization and trimethylsilylation without acidic hydrolysis. Sixteen and 12 modified bases were identified and quantified in DNA samples irradiated under anoxic and oxic conditions, respectively. These were 5,6-diHThy, 5,6-diHUra, 5-OH-5-MeHyd, 5-OH-Hyd, 5-OH-Ura, 5-OH-Cyt, 5-OH-6-HThy, 5-OH-6-HUra, 5-OHMeUra, Thy glycol, 5,6-diOH-Ura, FapyAde, 8-OH-Ade, 2-OH-Ade, FapyGua, and 8-OH-Gua. Under oxic irradiation conditions, 5,6-diHThy, 5,6-diHUra, 5-OH-6-HThy, and 5-OH-6-HUra were not observed. These results were in agreement with our previously published results (Fuciarelli et al., 1990; Gajewski et al., 1990). Calf thymus DNA used in the present study contained some of the modified bases at detectable levels.

The uracil derivatives 5,6-diHUra, 5-OH-Hyd, 5-OH-Ura, 5-OH-6-HUra, and 5,6-diOH-Ura detected in hydrolysates in DNA are products resulting from •OH attack on cytosine as previously reported (Téoule & Cadet, 1978; Dizdaroglu & Simic, 1984; Dizdaroglu, 1985; Dizdaroglu et al., 1986; Téoule, 1987). In the present work, this was confirmed by GC/MS analysis of cytosine, which was γ-irradiated in aqueous solution under anoxic conditions. 5,6-diHUra, 5-OH-Ura, 5-OH-Cyt, 5-OH-6-HUra, Ura glycol, Cyt glycol, alloxan, 5,6-diOH-Ura, and 5,6-dihydroxycytosine (5,6-diOH-Cyt) were identified. Upon treatment of irradiated cytosine with formic acid similar to DNA hydrolysis, Ura glycol and Cyt glycol were converted into 5-OH-Ura and 5-OH-Cyt, in agreement with previously published results (Dizdaroglu et al., 1986). Alloxan and 5,6-diOH-Cyt were converted into 5-OH-Hyd and 5,6-diOH-Ura, respectively (data not shown). These results confirm that uracil derivatives observed in DNA pellets after acidic hydrolysis are products of the cytosine moiety. Some or all of these uracil derivatives may exist in DNA as their cytosine-derived analogues. Alloxan is known to be formed by spontaneous oxidation of dialuric acid [5-hydroxy-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione] in aqueous solution (Behrend & Roosen, 1889; Dizdaroglu, 1993). This means that dialuric acid [or its cytosine-derived analogue 4-amino-5-hydroxy-2,6(1*H*,5*H*)-pyrimidinedione] may be the primary product formed in DNA. Because of the spontaneous oxidation of dialuric acid, however, alloxan is likely to be the actual product prevalent in DNA. This is supported by the fact that, in the present work, alloxan was detected in supernatant fractions, which underwent no acidic treatment. Under the conditions of acidic hydrolysis of DNA, alloxan undergoes decarboxylation and is converted into 5-OH-Hyd (Dizdaroglu, 1993). The latter compound was detected in hydrolyzed DNA pellets.

The results obtained with quantification of pyrimidine-derived lesions excised by endonuclease III from DNA samples γ-irradiated under anoxic conditions are illustrated in Figure 1. The excision of 5,6-diHThy, 5,6-diHUra, 5-OH-5-MeHyd, alloxan, 5-OH-6-HThy, 5-OH-6-HUra, Thy glycol, and Ura glycol (detected as 5-OH-Ura) was demonstrated by the presence of these modified bases in the supernatant fractions of irradiated DNA samples incubated with the native enzyme (parts A, B, C, D, E, F, G, and H, respectively, of Figure 1). It should be pointed out that the amounts found in the supernatant fractions corresponded to the amounts removed from the DNA (Figure 1). 5-OH-Ura detected in supernatant fractions may have resulted from dehydration of Ura glycol (or from deamination and dehydration of Cyt glycol), as was discussed previously [see also Dizdaroglu et al. (1986)]. This means that Ura glycol (or Cyt glycol), not 5-OH-Ura, has been excised by the enzyme. 5-OH-5-MeHyd, alloxan, and Thy glycol were detected in all supernatant fractions (parts C, D, and G, respectively, of Figure 1). However, the amounts of these products in supernatant fractions of the samples incubated with the native enzyme were significantly higher than those in supernatant fractions of samples incubated without the enzyme or with the inactivated enzyme. After correction for background, these amounts corresponded to the amounts excised from DNA. The remaining three pyrimidine-derived lesions 5-OHMeUra, 5,6-diOH-Ura, 5-OH-Cyt and all purine-derived lesions were not excised by the native enzyme (data not shown). Figure 2 illustrates the results obtained with DNA samples γ-irradiated under oxic condi-

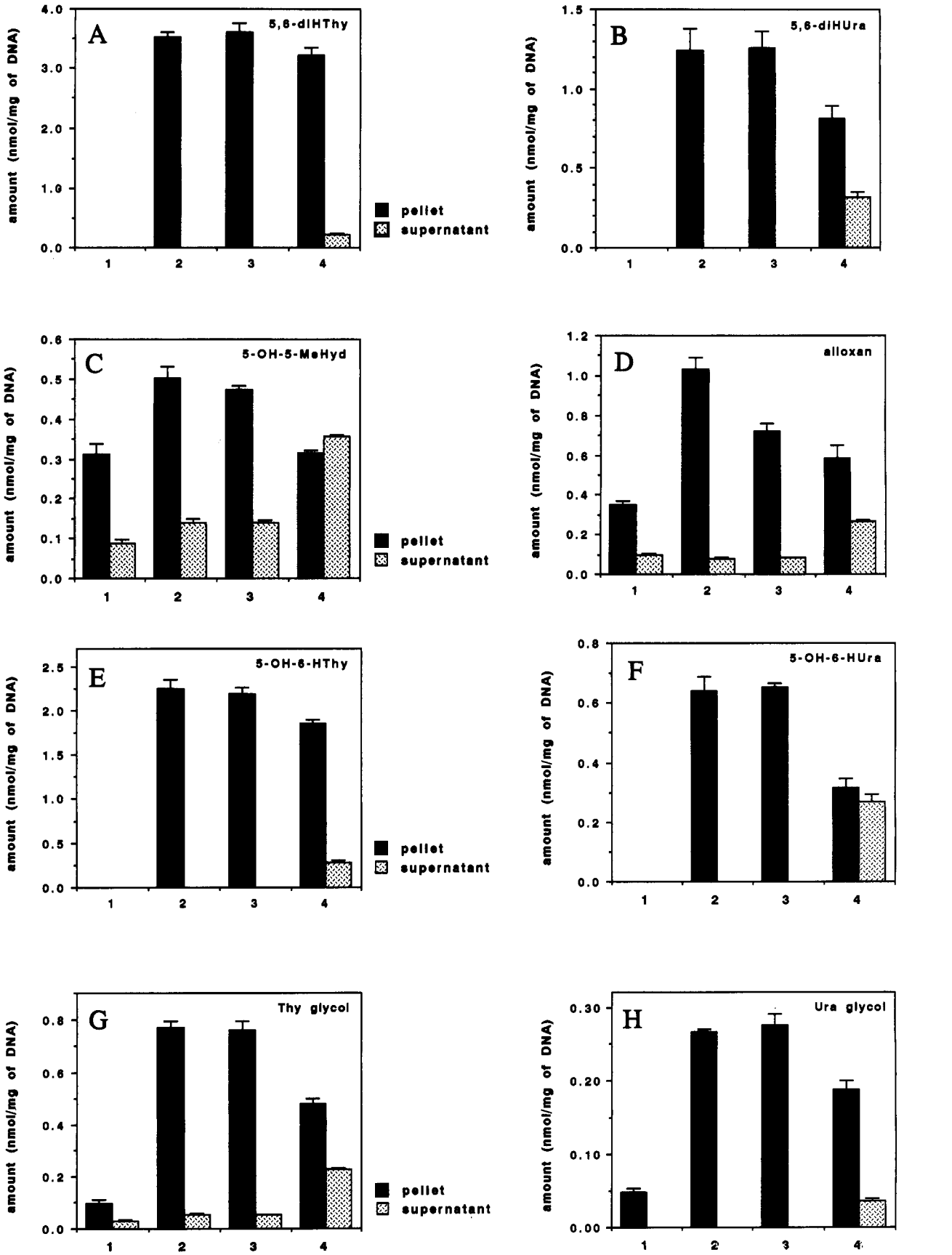

FIGURE 1: Yields of pyrimidine-derived lesions in pellets and supernatant fractions of DNA samples γ-irradiated under anoxic conditions (1 nmol of a modified base/mg of DNA ≈ 320 modified base residues/10^6 DNA base residues). 1, Unirradiated DNA; 2, γ-irradiated DNA; 3, γ-irradiated DNA after incubation with the inactivated endonuclease III; 4, γ-irradiated DNA after incubation with the native endonuclease III.

tions. 5-OH-5-MeHyd, alloxan, Thy glycol, and Ura glycol were excised by the native enzyme. Again, no excision of 5-OHMeUra, 5,6-diOH-Ura, 5-OH-Cyt, and purine-derived lesions was observed. Table I shows the percentage of excision

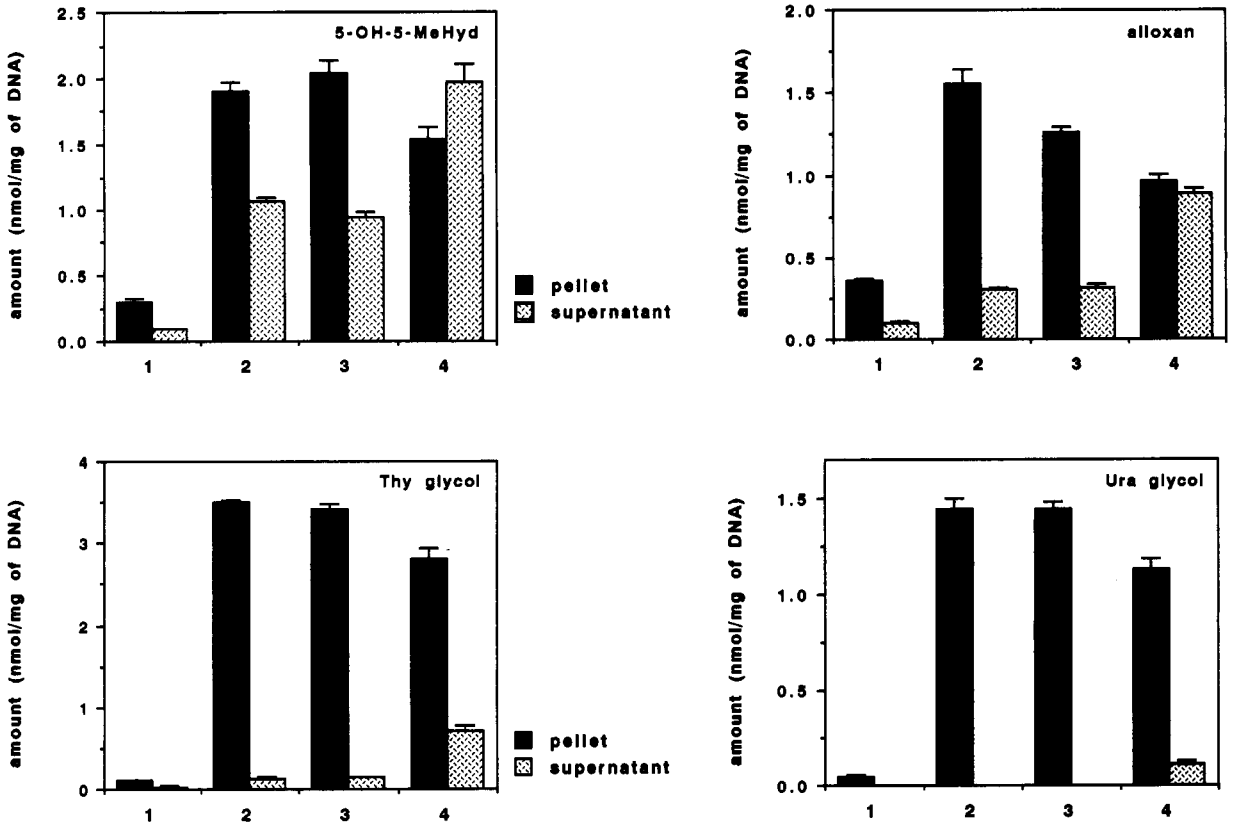

FIGURE 2: Yields of pyrimidine-derived lesions in pellets and supernatant fractions of DNA samples γ-irradiated under oxic conditions (1 nmol of a modified base/mg of DNA ≈ 320 modified base residues/10^6 DNA base residues). 1, Unirradiated DNA; 2, γ-irradiated DNA; 3, γ-irradiated DNA after incubation with the inactivated endonuclease III; 4, γ-irradiated DNA after incubation with the native endonuclease III.

Table I: Percentage[a] of Excision of Pyrimidine-Derived Lesions by Endonuclease III from γ-Irradiated DNA

	irradiation conditions	
product	anoxic	oxic
5,6-diHThy	6	
5-OH-5-MeHyd	44	47
5-OH-6-HThy	13	
Thy glycol	22	16
5,6-diHUra	25	
alloxan	18	38
5-OH-6-HUra	42	
Ura glycol[b]	14	8

[a] Values were calculated after correction for background amounts found in supernatant fractions of DNA samples incubated in buffer without the native enzyme. [b] Detected as 5-OH-Ura.

by the native enzyme of pyrimidine-derived lesions from both DNA substrates. The structures of the pyrimidine-derived lesions excised by endonuclease III are illustrated in Figure 3.

DISCUSSION

The results of the present study show that *E. coli* endonuclease III excises a number of pyrimidine-derived DNA lesions formed by ionizing radiation-generated free radicals under anoxic or oxic conditions. Our results confirm previously described excision of 5,6-diHThy, 5-OH-5-MeHyd, and Thy glycol. We also provide for the first time evidence for the ability of endonuclease III to recognize and excise another thymine-derived lesion and four cytosine-derived lesions. These are 5-OH-6-HThy, 5,6-diHUra, alloxan, 5-OH-6-HUra, and Ura glycol. The excision by endonuclease III of 5-OH-6-HThy has been proposed previously with no evidence provided (Breimer & Lindahl, 1985). The present study extends the substrate specificity of endonuclease III to a number of cytosine-derived lesions. Although cleavage of irradiated DNA at cytosine sites has been described previously, the chemical nature of lesions has not been elucidated (Helland et al., 1986).

As was discussed above, the uracil derivatives 5,6-diHUra, alloxan, 5-OH-6-HUra, and Ura glycol may exist in DNA as their cytosine-derived analogues. Thus, endonuclease III may have removed the cytosine derivatives rather than the uracil derivatives. However, the possibility exists that the cytosine derivatives underwent spontaneous deamination prior to enzymatic digestion of DNA, and thus the enzyme excised the uracil derivatives. From our results, we cannot conclude whether cytosine derivatives or uracil derivatives (or both simultaneously) were prevalent in DNA prior to enzymatic treatment. Of these uracil derivatives, Ura glycol is believed to be the precursor of 5-OH-Ura, which was detected in supernatant fractions of the samples treated with the native enzyme. Ura glycol may have dehydrated during derivatization to give 5-OH-Ura. No excision of 5-OH-Cyt, which may result from dehydration of Cyt glycol, was observed. 5-OH-Cyt may have been prevalent in DNA prior to enzymatic digestion. 5-OH-Ura and 5-OH-Cyt exist in their enol (unsaturated) forms (Behrend & Roosen, 1889; Stimson, 1949; Moschel & Behrman, 1974), and thus they would not be substrates for endonuclease III. The substrates of endonuclease III have been described to be ring-saturated, ring-contracted, or ring-fragmented pyrimidines (Breimer & Lindahl, 1985).

In the present work, 5,6-diHThy was excised by endonuclease III to a small extent. Demple and Linn (1980) tentatively showed the excision of this compound from OsO_4-

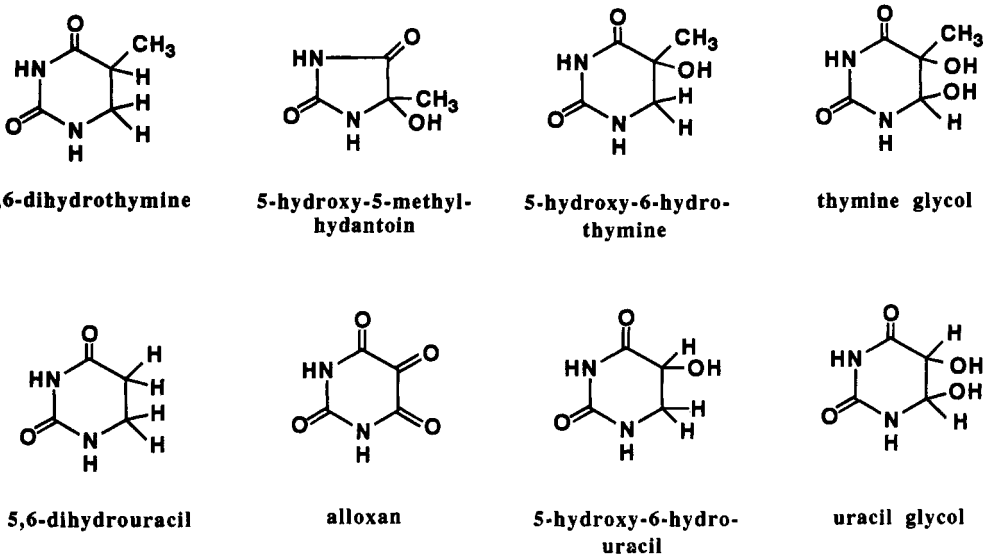

FIGURE 3: Structures of pyrimidine-derived lesions excised by endonuclease III. Uracil derivatives illustrated here may exist in DNA as their cytosine-derived analogues.

treated DNA. However, the following work did not confirm the formation of 5,6-diHThy in OsO_4-treated DNA (Dizdaroglu et al., 1986). On the other hand, 5,6-diHUra (or 5,6-diHCyt) was excised substantially more than 5,6-diHThy. These two compounds are produced by reactions of H atom and e_{aq}^- with cytosine and thymine, respectively [for a review see von Sonntag (1987)].

The thymine-derived lesion 5-OHMeUra was not excised by endonuclease III. This compound is excised from DNA in mammalian cells by a specific DNA glycosylase (Hollstein et al., 1984; Cannon-Carlson et al., 1989). The cytosine-derived lesion 5,6-diOH-Ura, the excision of which was investigated here for the first time, was found not to be a substrate for endonuclease III. This compound may result from 5,6-diOH-Cyt by deamination or from isodialuric acid [6-hydroxy-2,4,5(1*H*,3*H*,6*H*)-pyrimidinetrione] by enolization, both of which have been identified previously as products of cytosine [for reviews see Téoule and Cadet (1978) and Téoule (1987)]. We do not know which of these forms is prevalent in irradiated DNA. Isodialuric acid exists in its keto form (Behrend & Roosen, 1889).

The purine lesions induced by ionizing radiation were not excised by endonuclease III. Another DNA glycosylase in *E. coli*, the Fpg protein, excises formamidopyrimidines and 8-hydroxypurines from DNA (Chetsenga & Lindahl, 1979; Breimer, 1984; Boiteux et al., 1984, 1992; Tchou et al., 1991). Excision of 2-OH-Ade by a DNA glycosylase has not been reported.

In conclusion, the present study provides evidence for the excision of a number of thymine- and cytosine-derived lesions and thus extends the substrate specificity of *E. coli* endonuclease III. The chemical nature of these products is in agreement with the current hypothesis, which suggests that endonuclease III initiates the repair of pyrimidine-derived lesions through the recognition of the loss of the 5,6-double bond, resulting in the nonplanar structure of such moieties (Katcher & Wallace, 1983; Breimer & Lindahl, 1984). The results support the view that the pyrimidine-specific endonuclease III may complement the purine-specific Fpg protein in the repair of oxidative DNA damage.

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